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14. ABSTRACT
A variety of unique metal mixtures have entered the military arsenals of many countries in recent years. One such material is the tungsten alloys, which have been proposed as replacements for depleted uranium (DU) in armor-penetrating munitions. As a result, opportunities for exposure are increasingly likely. This leads to questions, similar to those originally surrounding DU, as to the health effects of exposure to the tungsten alloys, especially for embedded fragment exposures. The Armed Forces Radiobiology Research Institute (AFRRI) recently performed research that showed one of the militarily promising tungsten alloys to be a potent carcinogen when implanted in rats. The need to confirm the carcinogenicity of such alloys in another rodent species is an important second step required in biological as well as regulatory terms to better assess the cancer risk in humans. Results of this work will help in formulating policies for military surgeons who must treat personnel wounded by fragments of the alloys. Indications of unacceptable risks of exposure will also help determine the advisability of deploying (or developing) similar munitions. In year 2 of this project, despite a change in Principal Investigator, substantial progress has been made. Pellets for implantation were received and all mice in the 24-month experimental groups successfully implanted. At present, there have been no adverse health effects as a result of pellet implantation.

15. SUBJECT TERMS
Tungsten alloy; carcinogenicity; munitions; mice

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INTRODUCTION

A variety of unique metal mixtures have entered the military arsenals of many countries in recent years. One such material is the tungsten alloys, which have been proposed as replacements for depleted uranium (DU) in armor-penetrating munitions. As a result, opportunities for exposure are increasingly likely. This leads to questions, similar to those originally surrounding DU, as to the health effects of exposure to the tungsten alloys, especially for embedded fragment exposures. The Armed Forces Radiobiology Research Institute (AFRRI) recently performed research that showed one of the militarily promising tungsten alloys to be a potent carcinogen when implanted in rats. The need to confirm the carcinogenicity of such alloys in another rodent species is an important second step required in biological as well as regulatory terms to better assess the cancer risk in humans. Results of this work will help in formulating policies for military surgeons who must treat personnel wounded by fragments of the alloys. Indications of unacceptable risks of exposure will also help determine the advisability of deploying (or developing) similar munitions. The National Toxicology Program (NTP) Two-Year Study Protocol carried out in two rodent species is the recommended approach in the U.S. for identifying human carcinogens. This investigation aims to confirm the previous AFRRI data in rats by carrying out a two-year protocol in mice based upon NTP guidelines. The study uses the B6C3F1 hybrid mouse, a strain commonly used in carcinogenicity and toxicity assessment studies, implanted with pellets of tungsten alloys, the individual component metals of the alloys, tantalum (negative control), or nickel (positive control). The protocol includes serial collection of tissues 1, 3, 6, and 12 months post-implantation aimed at identifying early changes relevant to the development of carcinogenic endpoints.

BODY

The original Principal Investigator (PI) of this project, Dr. David McClain, abruptly retired in April 2007. Application was made to the Peer-Reviewed Medical Research Program (PRMRP) at that time requesting a change in PI and consideration of a revised statement of work to more adequately address the research goals within the allotted budget. This request was approved on July 17, 2007 and Dr. John Kalinich assumed the role of Project PI. The approved revised statement of work is below.

Hypothesis and Aims

AFRRI research recently showed that mixtures of tungsten, nickel, and cobalt are tumorigenic and genotoxic in HOS cells and that embedded pellets of the alloy tungsten-nickel-cobalt cause cancer in rats. However, studies with cultured cells and rats are not in themselves sufficient to allow designation of a substance as carcinogenic in humans. In general, the National Toxicology Program (NTP) and the Environmental Protection Agency (EPA), two agencies involved in cancer risk determination, agree that convincing evidence that the agent is probably carcinogenic in humans is obtained if the agent demonstrates carcinogenic potential in two rodent species, using the NTP two-year carcinogenicity protocol. This study proposes to obtain that data.

We <u>hypothesize</u> that the alloys tungsten/nickel/cobalt and tungsten/nickel/iron are carcinogenic in the mouse as indicated by the NTP two-year carcinogenicity protocol. Our test of this hypothesis will incorporate the following Aims.

Aim 1: Apply the NTP two-year carcinogenicity protocol to determine whether the alloys tungsten/nickel/cobalt and tungsten/nickel/iron cause cancer in mice. Include in the protocol mice embedded with pellets of the individual metals composing the alloys and the various metal combinations (blended with biologically inert tantalum at the same percentages present in the alloys).

Aim 2: Sacrifice mice at various times after alloy implantation to detect early signs of tumor development.

Aim 3: Measure tissue levels of the various metals that compose the alloys to correlate metals levels with tumor development.

Technical Objectives

Aim 1: Determine whether the alloys tungsten-nickel-cobalt and tungsten-nickel-iron cause cancer in mice. Include in the protocol mice embedded with pellets of the individual metals composing the alloys and the various metal combinations (blended with biologically inert tantalum at the same percentages present in the alloys).

Pellets of the alloys or the various component metals will be implanted in the leg muscles of mice, and mice will be maintained and monitored for 24 months post-implantation. The response in alloy-implanted mice will be compared to mice implanted with pellets of 100% nickel, a known carcinogen (positive controls) and mice implanted with tantalum, an inert metal used in prosthetic devices (negative control). At the end of the study or whenever sacrifice of participating mice is required, necropsies will be performed to obtain evidence of tumor development. Data of tumor sites and incidence will be compiled. Tumors will be histologically examined and classified.

Aim 2: Sacrifice mice at various times after alloy implantation to detect early signs of tumor development.

Subgroups of animals treated identically to the mice described in Aim 1 will be euthanized 1, 3, 6, and 12 months after metal implantation to identify any early signs of histopathology associated with exposure to the implanted metals.

Aim 3: Measure tissue levels of the various metals that compose the alloys to correlate metals levels with tumor development.

Levels of W, Ni, Fe, Co, and Ta will be measured in organs from the animals used in Aims 1 and 2. Data will be used to relate tissue metal levels to any cancer incidence observed in those particular tissues. These data will allow a correlation of tissue metal content with tumor development.

Project milestones

The following milestones will be met for the four years proposed for this study.

Year 1

a. Contract for manufacture of pellets required for project

Year 2

a. Implant 24-month exposure animals (560 mice)
b. Begin implantation of 1-month exposure animals (150 mice)
c. Begin necropsy, histopathology, and clinical assessment of 1-month animals

Year 3

a. Complete implantation of 1-month exposure animals
b. Complete necropsy, histopathology, and clinical assessment of 1-month animals
c. Implant 3-month exposure animals (150 mice)
d. Necropsy, histopathology, and clinical assessment of 3-month animals
e. Implant 6-month exposure animals (150 mice)
f. Implant 12-month exposure animals (150 mice)
g. Compile experimental data on 1- and 3-month animals

Year 4

a. Necropsy, histopathology, and clinical assessment of 6-month animals
b. Necropsy, histopathology, and clinical assessment of 24-month animals
c. Necropsy, histopathology, and clinical assessment of 12-month animals
d. Compile experimental data on 6-, 12-, and 24-month animals
e. Analyze all data
f. Provide final report

Methods and Experimental Design

The research proposed is based on the guidelines of the NTP Two-Year Study Protocol for assessing potential carcinogens. The guidelines suggest the use of two rodent species; specifically, common strains of rat (e.g., Fisher 344) and/or mouse (e.g., B6C3F1) are recommended, unless overriding considerations such as special susceptibility to a particular compound or route of exposure dictate use of other animals. Rats and mice are among the easiest laboratory animals to maintain, and there is a wealth of physiological data available to help guide such studies. AFRRI has already shown that embedded pellets of a tungsten/nickel/cobalt alloy cause cancer in the Fisher 344 rat employed in an NTP-style study. This study will attempt to replicate that study in the B6C3F1 mouse.

The NTP and EPA guidelines for testing the potential carcinogenicity of an agent in rodents suggest that 50 males and 50 females be used for every treatment, that three doses be tested, and that a histopathological/clinical assessment be performed on animals leaving the study. Strict adherence to those standards would require funding far in excess of that available, so this project will carry out a study similar to the previous AFRRI study in rats that approaches the recommendations of the guidelines. We propose the use of fewer animals per test with only males being tested, two tungsten alloy pellet doses instead of three, and histopathological/clinical assessments initially only on subsets of treatment groups. The study does not propose reducing or eliminating treatments such as the testing of individual component metals, because data in that area could produce results helpful in understanding mechanisms of carcinogenicity and assist to weapons developers choosing metals for munitions design. Included in the protocol will be additional mice to be sacrificed at various times during the course of exposure to assess changes associated with early tumor development.

The B6C3F1 mouse will be used for these experiments. The hybrid B6C3F1 mouse is commonly used in a wide variety of research applications, particularly toxicology. A study employing 1200 mice will provide a sufficient number to perform the described two-year carcinogenicity assessment protocol using fifteen treatment groups, serial sacrifices to test for early changes in exposed animals, and sufficient additional animals to serve as colony sentinels and backups.

The project will focus on two tungsten alloys of special interest to the military: 91.1% tungsten/6% nickel/2.9% cobalt and 91% tungsten/7% nickel/2% iron. All of the tests proposed will include fifteen treatment groups consisting of various controls, tungsten alloy metal tests, and a toxicity reference metal (lead). Exposures will be accomplished by implantation of the metals as pellets in the form of cylinders 1 mm in diameter and 2 mm long. Alloy pellets will be custom-fabricated using sintered metal powder technology similar to that used for military munitions. Pellets designed to test individual metals in the alloys will contain the same percentage content by weight as the full alloy, with the balance made up with the biologically inert metal tantalum. Lead and nickel pellets (toxicity reference metal and positive control, respectively) will be cut from wires of pure metal and formed to a dimension identical to the alloy pellets. The individual test groups are described as follows:

1. Sham-implantation controls
2. Tantalum pellet-implanted (implantation controls)
3. Nickel (100%) pellet-implanted (positive controls)
4. Lead (100%) pellet-implanted (reference metal)
5. Tungsten/nickel/cobalt pellet-implanted
6. Tungsten/nickel/iron pellet-implanted
7. Tungsten/tantalum pellet-implanted
8. Nickel/tantalum pellet-implanted
9. Cobalt/tantalum pellet-implanted
10. Iron/tantalum pellet-implanted
11. Tungsten/nickel/tantalum pellet-implanted
12. Tungsten/cobalt/tantalum pellet-implanted
13. Tungsten/iron/tantalum pellet-implanted
14. Cobalt/nickel/tantalum pellet-implanted
15. Iron/nickel/tantalum pellet-implanted

Two-Year Carcinogenicity Study: These experiments will test the carcinogenic potential of two doses (2 and 4 pellets) of pellets implanted in mice for 24 months. Twenty male mice will be used in each treatment group. The doses to be used and manner in which the animals are exposed are based on successful mouse and rat pellet implantation models designed at AFRRI and used for nearly a decade. Mice will be weighed on a weekly basis and observed for any changes indicative of developing pathology. At the end of the 24-month period or at any time mice appear moribund, they will be sacrificed. At the time of sacrifice, blood will be drawn for a complete hematological and clinical assessment, and the mice will undergo full necropsy, preserving selected tissues and organs and preparing slides for histopathological examination as required.

Serial Sacrifice Study: The serial sacrifice study will run in parallel with the two-year carcinogenicity study and will also include the fifteen treatments groups. Ten male mice will be employed in each treatment group. One, 3, 6, and 12 months after pellet implantation, mice will be sacrificed, and gross pathologies performed. Selected tissues will be collected and preserved and hematological tests performed. Histopathological surveys of selected animals will determine whether more extensive histopathology will be performed.

KEY RESEARCH ACCOMPLISHMENTS

- Change in Project Principal Investigator and revised Statement of Work was accepted by PRMRP.
- Pellets were manufactured and delivered by Aerojet Ordnance Tennessee.
- Pellet implantation procedures were modified to allow for a smaller incision and elimination of the need for extensive suturing of the implantation site. This refinement allowed animals to recover from the procedure more quickly with no complications.
- All animals in both the low- and high-dose 24 month groups (560 mice) have been successfully implanted and the surgical schedule for 1 month groups initiated.

REPORTABLE OUTCOMES

Oral Presentations

Kalinich, JF (23 March 07) Health Effects of Embedded Tungsten Alloy and Depleted Uranium. AFRRI Seminar. Bethesda, MD.

Kalinich, JF (29 May 07) Health Effects of Embedded Tungsten Alloy. Briefing to Deputy Undersecretary of Defense for Acquisition, Technology, and Logistics and Deputy Assistant Secretary of Defense for Force Health Protection and Readiness. Arlington, VA.

Kalinich, JF (24 July 07) Tissue Distribution Patterns of Tungsten Alloy Component Metals from Embedded Fragments. Presentation at the Force Health Protection Embedded Fragment Working Group Meeting. Falls Church, VA.

Kalinich, JF (06 November 07), Health Effects of Embedded Tungsten Alloy. Presentation at the Baltimore Veteran's Administration Medical Center / Toxic Embedded Fragment Center. Baltimore, MD.

Kalinich, JF (09 January 08), Health Effects of Embedded Tungsten Alloy. Presentation at the Baltimore Veteran's Administration Medical Center / Toxic Embedded Fragment Center's Expert Panel Meeting. Baltimore, MD.

CONCLUSION

Despite the unexpected departure of Dr. McClain and subsequent administrative changes, the project has made significant progress in attaining its stated aims. In this reporting year pellets for the implantation studies were received from Aerojet Ordnance Tennessee. Procedures to manufacture some of the novel metal mixtures to be tested required substantial metallurgic expertise to accomplish and the staff at Aerojet were outstanding in this regard. Also in this reporting year, all mice in the 24-month treatment groups were successfully implanted (560 mice total). General health assessments of the animals are conducted daily, with body weights and implantation site examination (including palpitation) conducted weekly. At present, there have been no adverse effects as the rest of pellet implantation. In addition, improvements in the surgical implantation procedure has allowed for a smaller incision with no suturing required resulting in shorter recovery times with no complications.

REFERENCES

None.

APPENDICES

None.